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FURTHER STUDIES ON THE *IN VIVO* EFFECT OF DIISOPROPYL 1,3-DITHIOL-2-YLIDENEMALONATE (NKK-105) ON THE LIVER MICROSOMAL DRUG OXIDATION SYSTEM IN RATS

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Abstract—The effect of diisopropyl 1,3-dithiol-2-ylidenemalonate (NKK-105) on microsomal electron transport systems in relation to drug oxidation was studied in rat liver. A single oral dose (250 mg/kg) of NKK-105 increased the ratio of liver to body weight, the microsomal protein content, the cytochrome b_5 content, and the NADPH cytochrome *c* reductase activity at 24–48 hr after drug administration. The cytochrome P-450 content was decreased at 2–6 hr and slightly increased at 24–48 hr after drug administration. Upon daily administration of NKK-105 at a dose of 250 $mg \cdot kg^{-1} \cdot day^{-1}$ for 21 days, cytochrome b_5 content and NADPH cytochrome *c* reductase activity were increased, but cytochrome P-450 content and NADH cytochrome b_5 reductase activity remained unchanged. Despite the increase of NADPH cytochrome *c* reductase activity, NADPH-dependent lipid peroxidation tended to decrease rather than increase. NADPH stearoyl-CoA desaturase activity increased prior to the increase of cytochrome b_5. Benzphetamine *N*-demethylase and *p*-nitroanisole *O*-demethylase activities were enhanced, accompanied by an increase of cytochrome b_5. Aniline hydroxylase activity was decreased by NKK-105 administration. These results indicate that the induction pattern of liver microsomal electron transport systems by NKK-105 is characteristic.

Diisopropyl 1,3-dithiol-2-ylidenemalonate (NKK-105) has various pharmacological effects on mammals. Nakayama *et al.* reported that NKK-105 increased the flow rate of hepatic blood and bile [1] and showed protective action against fatty liver induction by carbon tetrachloride [2]. It has also been reported that NKK-105 protects against liver injury induced by allyl alcohol, bromobenzene, carbon tetrachloride, chloroform, dimethylnitrosoamine and thioacetamide [3]. It was further found to have a therapeutic effect on fatty liver induced by carbon tetrachloride [4]. Indeed, Igarashi *et al.* [5] reported that NKK-105 increased the regeneration rate of partially hepatectomized cirrhotic rat liver.

NKK-105 has been reported to modify the activity of the hepatic microsomal mixed function monooxygenase system. Nakayama [6] demonstrated that NKK-105 increased the cytochrome P-450 and b_5 contents and drug oxidation activities in rat liver microsomes and caused changes in the fine structure of hepatocytes similar to those induced by phenobarbital. Our previous study showed that NKK-105 mainly induced microsomal cytochrome b_5 and NADPH cytochrome *c* reductase activity in rat liver, and that its induction pattern was quite different from those of phenobarbital, 3-methylcholanthrene, and polychlorinated biphenyl [7].

This paper presents the results of further work on the *in vivo* effect of NKK-105 on the hepatic microsomal drug oxidation system in rats.

MATERIALS AND METHODS

Animals and treatment.

Male Sprague–Dawley rats weighing 160–200 g were used. They were fed laboratory chow (Nihon Clea Co., Ltd., Tokyo, Japan, CE-2) and water *ad lib.* NKK-105, synthesized and recrystallized by the Nihon Nohyaku Co., Ltd. (Osaka, Japan) was dissolved in olive oil (5%, w/v) and administered orally at a dose of 250 mg/kg (1 ml/200 g body weight) once a day. Control rats received equivalent volumes of olive oil. Animals were killed at selected intervals after a single administration. In the case of repeated treatment with NKK-105, animals were killed 24 hr after the final administration. In all experiments except for stearoyl-CoA desaturase determination, animals were fasted for 18 hr prior to killing, but had free access to tap water.

Preparation of hepatic microsomes

Animals were killed by cervical dislocation and the livers were excised, weighed, perfused with ice-cold 1.15% KCl solution as quickly as possible, and homogenized in 4 vol. of the KCl solution. The

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homogenate was centrifuged at 9000 *g* for 20 min to remove nuclear and mitochondrial fractions. The microsomal pellet was obtained by further centrifugation at 105,000 *g* for 60 min. After being washed once with the KCl solution, the pellet was suspended in 0.1 M sodium–potassium phosphate buffer (pH 7.4) to give a protein concentration of about 10 mg/ml.

Biochemical assays

Cytochrome b_5 [8], cytochrome P-450 [8], NADH cytochrome b_5 reductase [9] and NADPH cytochrome *c* reductase [10] activities were determined according to the cited methods. Lipid peroxidation was measured in terms of the formation of malondialdehyde according to the method of Kamataki *et al.* [11], and the activity was expressed as optical density at 535 nm per ml of the reaction mixture per 15 min. Stearoyl-CoA desaturase activity was determined by measuring the oleate produced in the Δ^9-desaturation of [1-^{14}C]stearoyl-CoA (New England Nuclear Corp., Boston, MA, U.S.A.; 53.97 mCi/mmole) according to the method of Oshino *et al.* [12]. *p*-Nitroanisole *O*-demethylase activity was determined by the method of Kinoshita *et al.* [13] using the 9000 *g* supernatant fraction as an enzyme source. *N*-Demethylase activity toward aminopyrine, benzphetamine or ethylmorphine was determined by measuring the formaldehyde formed according to the method of Nash [14]. Aniline hydroxylase activity was estimated by measuring the *p*-aminophenol formed by the method of Imai *et al.* [15]. Unless otherwise mentioned, the incubation mixture consisted of microsomes (approximately 1 mg protein), sodium–potassium phosphate (64 mM, pH 7.4), EDTA (0.1 mM), an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$, and 0.113 units of glucose-6-phosphate dehydrogenase) and a substrate (0.1 M) in a final volume of 1.0 ml. The reaction was allowed to proceed for 15 min at 37° and was stopped by the addition of 10% trichloroacetic acid. Protein concentration was determined by the method of Lowry *et al.* [16].

Statistical analysis

The significance of the difference between two mean values was assessed by the use of Student's *t*-test.

RESULTS

Single treatment studies

The effects of a single administration of NKK-105 on the ratio of liver to body weight and on the components of the hepatic microsomal electron transport system were studied.

Changes in the ratio of liver to body weight and in the liver microsomal protein content after a single administration of NKK-105. As shown in Fig. 1, the ratio of liver to body weight and the microsomal protein content gradually increased and reached maximum values at 24 hr after NKK-105 treatment.

Changes in cytochrome b_5 *and P-450 contents.* Figure 2 shows that cytochrome b_5 content was significantly enhanced at 24–48 hr after drug treatment and thereafter gradually declined (Fig. 2A). Conversely, cytochrome P-450 content appears to have diminished at 2–6 hr, and then to have been enhanced at 24–48 hr after dosing, but the difference between the NKK-105-treated and control groups was not statistically significant. The ratios of cytochrome P-450 to cytochrome b_5 contents in the control and NKK-105-treated groups were 2.06 and 1.81 respectively.

Changes in NADH cytochrome b_5 *reductase and NADPH cytochrome c reductase activities after a single administration of NKK-105.* The changes in the two reductase activities are shown in Fig. 3. At 24 hr after the administration of NKK-105, NADH cytochrome b_5 reductase (Fig. 3A) appeared to have increased slightly, but this was not statistically significant. This increment was not observed when the activity was expressed per mg of protein. Six hours after the drug administration, NADPH cytochrome *c* reductase (Fig. 3B) began to rise. It reached the maximum value, 200 per cent of the control level, at 24 hr and then gradually declined.

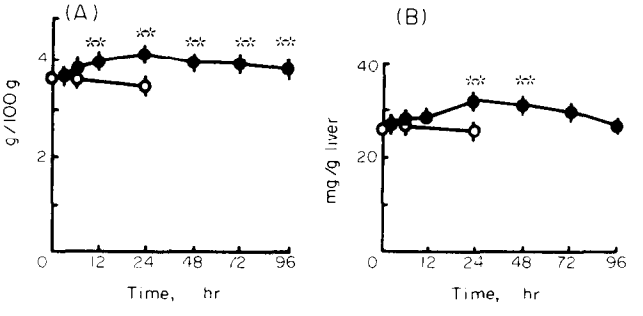

Fig. 1. Changes in the liver to body weight-ratio (×100) (A) and liver microsomal protein content (B) after administration of NKK-105 to rats. Numbers (g/100g) indicate ratio of liver to body weight, (g/g ×100). In all figures in this paper, NKK-105 was orally administered at a dose of 250 mg/kg to rats and liver microsomes were prepared at intervals. Key: (○) control group, and (●) NKK-105-treated group. Each point is the mean value of four rats. Vertical lines represent S.E. Double asterisks indicate $P < 0.01$ vs control.

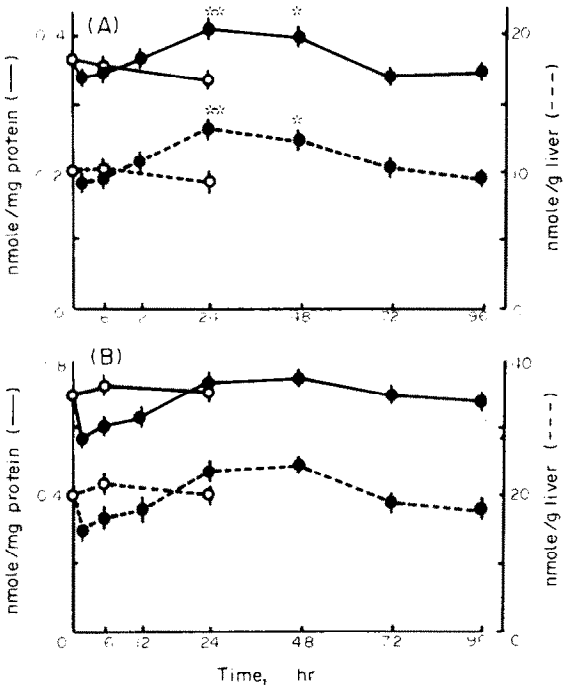

Fig. 2. Changes in the contents of microsomal cytochrome b_5 (A) and cytochrome P-450 (B) in rat liver after administration of NKK-105. A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. Key: (○) control group, and (●) NKK-105 -treated group. For details, see the legend to Fig. 1.

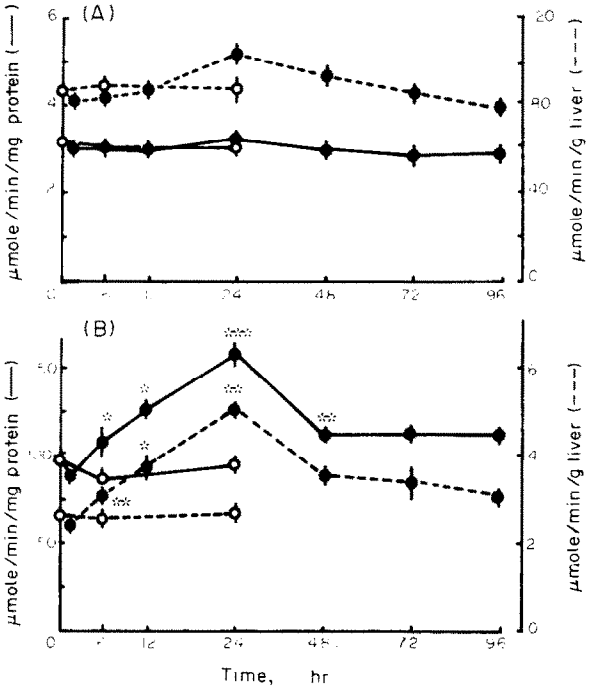

Fig. 3. Changes in the activities of microsomal NADH cytochrome b_5 reductase (A) and NADPH cytochrome *c* reductase (B) in rat liver after administration of NKK-105. Key: (○) control group, and (●) NKK-105-treated group. A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control; triple asterisks indicate $P < 0.001$ vs control. For details, see legend to Fig. 1.

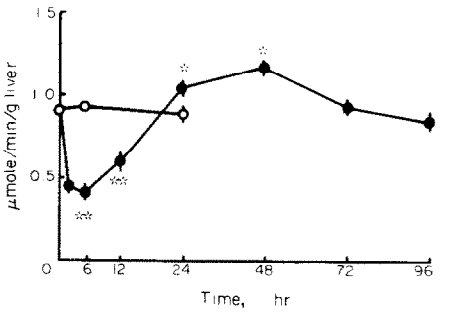

Fig. 4. Changes in the activity of *p*-nitroanisole *O*-demethylase in rat liver after administration of NKK-105. NKK-105 was orally administered at a dose of 250 mg/kg to rats and 9000 *g* supernatant fluid instead of microsomes of the liver was prepared at intervals. Key: (○) control group, and (●) NKK-105-treated group. A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. For details, see the legend to Fig. 1.

Changes in the activity of p-*nitroanisole* O-*demethylase after a single administration of NKK-105.* *p*-Nitroanisole *O*-demethylase activity decreased to 50 per cent of control level within 2–12 hr and then increased to approximately 150 per cent of control value within 24–48 hr after NKK-105 treatment (Fig. 4).

Repeated administration studies

Changes in the components of the microsomal electron transport system in rat liver upon daily administration of NKK-105 for 21 days. Table 1 shows the changes in the levels of components of the liver microsomal electron transport system after repeated administration of NKK-105. Microsomal protein and cytochrome P-450 contents and NADH cytochrome b_5 reductase activity remained unchanged. The ratio of liver to body weight, and the NADPH cytochrome *c* reductase and *p*-nitroanisole *O*-demethylase activities were increased at most of the time points investigated. Cytochrome b_5 increased continuously and reached about 170 per cent of control level on day 7 and 200 per cent on days 14 and 21.

The daily administration of NKK-105 was discontinued and the recovery of components of the microsomal electron transport system affected by NKK-105 was studied for the next 14 days. All the components studied here recovered to the control levels within 14 days.

Effect of NKK-105 administration on microsomal lipid peroxidation and stearoyl-CoA desaturase activities in rat liver. NKK-105 was administered to rats as a single dose or by repeated treatment for 4 days. Liver microsomes were prepared 4, 24, and 48 hr after single administration or 24 hr after the final administration in the repeated dose experiment. Lipid peroxidation and stearoyl-CoA desaturase activities were determined and the results are summarized in Table 2. NADPH-dependent lipid peroxidation was slightly decreased 24 hr after a single administration of NKK-105, and a distinct decrease was observed after repeated administration. Previously, NADPH-dependent lipid peroxidation was reported to be dependent on NADPH cytochrome *c* reductase activity [17]. In the present study, despite

Table 1. Effect of NKK-105 on microsomal electron transport components in rat liver*

Item	NKK-105 treatment	Administration period of NKK-105 (days)				
		2	4	7	14	21
Ratio of liver	Control	3.52 ± 0.09	3.52 ± 0.02	3.45 ± 0.08	3.17 ± 0.04	3.20 ± 0.08
to body weight ×100	Treated	4.27 ± 0.06†	4.12 ± 0.08†	4.35 ± 0.05†	4.06 ± 0.12†	4.59 ± 0.02†
Microsomal protein	Control	28.1 ± 3.1	32.6 ± 1.3	33.0 ± 0.6	32.1 ± 2.3	31.1 ± 1.3
(mg/g liver)	Treated	26.6 ± 0.7	33.7 ± 0.4	31.5 ± 0-4	37.2 ± 1.6	32.1 ± 0.9
Cytochrome b_5	Control	9.98 ± 0.70	9.68 ± 0.50	8.78 ± 0.40	10.76 ± 0.20	11.52 ± 0.34
(nmoles/g liver)	Treated	13.22 ± 0.88‡	13.52 ± 0.86§	15.10 ± 0.38†	21.62 ± 0.90†	23.10 ± 2.32§
Cytochrome P-450	Control	22.07 ± 2.65	17.06 ± 0.74	23.55 ± 1.96	23.54 ± 1.98	22.85 ± 1.45
(nmoles/g liver)	Treated	18.64 ± 0.99	18.36 ± 0.82	22.53 ± 1.94	27.34 ± 1.89	26.70 ± 1.60
NADH cytochrome b_5 reductase	Control	120.1 ± 19.8	102.4 ± 5.3	109.7 ± 6.5	95.50 ± 6.80	106.5 ± 8.2
[μmoles·min^{-1}·(g liver)$^{-1}$]	Treated	111.8 ± 3.0	93.9 ± 3.7	124.3 ± 7.2	128.0 ± 13.5	123.4 ± 8.0
NADPH cytochrome c reductase	Control	4.79 ± 0.62	3.18 ± 0.03	3.78 ± 0.16	3.47 ± 0.13	3.42 ± 0.35
[μmoles·min^{-1}·(g liver)$^{-1}$]	Treated	6.09 ± 0.17‡	4.54 ± 0.12†	6.82 ± 0.45	5.87 ± 0.23†	5.54 ± 0.41
p-Nitroanisole	Control	0.745 ± 0.128	0.702 ± 0.036	0.898 ± 0.078	1.014 ± 0.055	0.895 ± 0.063
O-demethylase	Treated	0.812 ± 0.011	0.889 ± 0.029§	1.037 ± 0.041	1.276 ± 0.053	1.205 ± 0.058‡
[μmoles·hr^{-1}·(g liver)$^{-1}$]						

* Each value is the mean ± S.E. from four rats. NKK-105 was orally administered at a dose of 250 mg·kg^{-1}·day^{-1} to rats, and liver microsomes were prepared 24 hr after the final administration. P values indicate the probability that the difference from control would have occurred by chance (Student's t-test).

† $P < 0.001$.

‡ $P < 0.05$.

§ $P < 0.01$.

Table 2. Effect of NKK-105 on microsomal lipid peroxidation and stearoyl-CoA desaturase activities in rat liver*

Treatment	Lipid peroxidation		Stearoyl-CoA desaturase	
Control	159.9 ± 13.8†	5.94 ± 0.55‡	13.19 ± 1.29§	0.49 ± 0.06‖
NKK-105, after 4 hr	149.2 ± 3.7	5.71 ± 0.21	21.49 ± 2.35	0.79 ± 0.07¶
24 hr	131.8 ± 4.8¶	4.40 ± 0.20	20.69 ± 3.00	0.84 ± 0.14¶
48 hr	143.4 ± 3.4	5.47 ± 0.25	22.39 ± 2.94¶	0.88 ± 0.13¶
for 4 days	102.3 ± 15.3¶	3.90 ± 0.20¶	29.01 ± 3.36**	1.09 ± 0.16¶

* Each value is the mean ± S.E. from four rats. NKK-105 was orally administered to rats at a dose of 250 $mg \cdot kg^{-1} \cdot day^{-1}$ (single dose or repeated administration for four days). Liver microsomes were prepared 4, 24, and 48 hr after a single administration or 24 hr after the final administration. P values indicate the probability that the difference from control would have occurred by chance (Student's *t*-test).

† O.D. 535 · 15 $min^{-1} \cdot (g\ liver)^{-1}$.

‡ O.D. 535 · 15 $min^{-1} \cdot (mg\ protein)^{-1}$.

§ $nmoles \cdot min^{-1} \cdot (g\ liver)^{-1}$.

‖ $nmoles \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

¶ $P < 0.05$.

** $P < 0.01$.

the significant increase in NADPH cytochrome *c* reductase (Table 1 and Fig. 3), NADPH-dependent lipid peroxidation tended to decrease. In this connection, it was expected that NADPH stearoyl-CoA desaturase activity would be increased by the administration of NKK-105, since NADPH stearoyl-CoA desaturase has been reported to be dependent on cytochrome b_5 [18]. As expected, the desaturase activity was enhanced within 4–48 hr after a single dose of NKK-105; moreover, it reached 220 per cent of control level after repeated administration for 4 days.

Effect of NKK-105 on microsomal drug oxidation activities in rat liver. The *N*-demethylase activities toward aminopyrine, benzphetamine, and ethylmorphine and aniline hydroxylase activity were determined. As shown in Table 3, *N*-demethylase activities toward aminopyrine and ethylmorphine were decreased significantly 4 hr after a single dose of NKK-105. Benzphetamine *N*-demethylase activity was found to be increased to 170 per cent of the control level 24 hr after NKK-105 administration. After repeated administration of NKK-105 for 4 days, *N*-demethylase activities toward aminopyrine, benzphetamine and ethylmorphine were increased to 227, 293 and 191 per cent of control level respectively. Aniline hydroxylase activity was reduced significantly within 24 hr after a single dose and fell to 76 per cent of control level after repeated administration of NKK-105.

DISCUSSION

The data in the present paper demonstrate that NKK-105 increases the ratio of liver to body weight, the liver microsomal protein content, the cytochrome b_5 content and the NADPH cytochrome *c* reductase activity 24–48 hr after a single administration of NKK-105 to rats. Cytochrome b_5 and NADPH cytochrome *c* reductase activity were 140 and 180 per cent, respectively, of the control activities 24 hr after the drug treatment. When NKK-105 was administered daily for 20 days, cytochrome b_5 content and NADPH cytochrome *c* reductase activity were enhanced at all the time points investigated. cytochrome b_5 increased continuously for 14 days

Table 3. Effect of NKK-105 on microsomal drug oxidation activities in rat liver*

Treatment	Aminopyrine *N*-demethylase	Benzphetamine *N*-demethylase	Ethylmorphine *N*-demethylase	Aniline hydroxylase
		[$nmoles \cdot min^{-1} \cdot (g\ liver)^{-1}$]		
Control	91.0 ± 0.6	173.6 ± 3.7	106.7 ± 1.7	25.9 ± 1.5
NKK-105, after 4 hr	72.2 ± 5.3†	128.0 ± 17.3	74.9 ± 7.7‡	9.6 ± 1.3§
24 hr	114.5 ± 4.8‡	300.8 ± 17.7	105.7 ± 6.7	12.9 ± 0.4§
48 hr	111.0 ± 8.4	256.0 ± 21.2	117.2 ± 10.4	21.5 ± 1.7
for 4 days	207.1 ± 15.4	510.3 ± 29.9	204.5 ± 11.3	19.6 ± 1.4

* Each value is the mean ± S.E. from four rats. NKK-105 was orally administered to rats at a dose of 250 $mg \cdot kg^{-1} \cdot day^{-1}$ (single dose or repeated administration for 4 days). Liver microsomes were prepared 4, 24 and 48 hr after a single administration or 24 hr after the final administration. P values indicate the probability that the difference from control would have occurred by chance (Student's *t*-test).

† $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.001$.

and then maintained the increased level until day 21; NADPH cytochrome *c* reductase activity was maintained at a higher level until 14. The reason for the difference between the changes in cytochrome b_5 and NADPH cytochrome *c* reductase may be that the half-life of cytochrome b_5 is longer than that of NADPH cytochrome *c* reductase [19]. Cytochrome P-450 content was reduced at 2–6 hr and slightly enhanced at 24–48 hr after a single dose of NKK-105. When NKK-105 was administered daily for 14 and 21 days, the cytochrome P-450 level scarcely increased. NADH cytochrome b_5 reductase activity was not affected by the administration of NKK-105. Recently, Nakayama reported that a single oral administration of NKK-105 at three doses (250, 500 and 1000 mg/kg) induced an apparent increase in liver weight, an elevation of aminopyrine *N*-demethylase activity and a slight increase in microsomal cytochrome b_5 and P-450 contents [6]. The results in this study are similar to those in Nakayama's paper.

Many xenobiotics such as drugs, pesticides, and other chemicals induce components of the microsomal electron transport system in mammalian liver [20–24]. Madhukar and Matsumura [25] investigated the changes in microsomal mixed function oxidase in rat liver caused by pesticides and related compounds, and they proposed that these compounds could be classified into several types according to the induction patterns. NKK-105, however, does not fall into any of the categories proposed by them. With regard to the induction of liver microsomal cytochrome b_5, griseofulvin [26] was reported to increase microsomal cytochrome b_5 content and NADH cytochrome b_5 reductase and NADPH cytochrome *c* reductase activities but to decrease cytochrome P-450 content in mouse liver, and AF-2 was reported to increase microsomal cytochrome b_5 content, but to decrease cytochrome P-450 content in rat liver [27]. In 1977, Kahl and Netter [28] reported that ethoxyquine, an antioxidant, enhanced microsomal cytochrome b_5 and P-450 contents to the same extent in rat liver. Our previous paper demonstrated that the induction pattern of the microsomal electron transport components in rat liver by NKK-105 is different from those shown by phenobarbital, 3-methylcholanthrene, and polychlorinated biphenyl [7].

In the liver microsomal electron transport system, three terminal oxidation activities are known: (1) lipid peroxidation, (2) stearoyl-CoA desaturation, and (3) drug oxidation. Stearoyl-CoA desaturase is known to be dependent on cytochrome b_5. Jansson and Schenkman [18] demonstrated that microsomal desaturase activity was increased in proportion to the amount of added detergent-isolated cytochrome b_5. In the present study, cytochrome b_5 content and stearoyl-CoA desaturase activity were increased to 150 and 220 per cent of the control levels, respectively, after daily administration of NKK-105 for 4 days. The increase in the desaturase activity induced by NKK-105 was higher than expected from the degree of increase of cytochrome b_5 content. This suggests that the protein content of desaturase may be increased by NKK-105. Lipid peroxidation is dependent on NADPH cytochrome *c* reductase [17]. Despite the increase in NADPH cytochrome *c* reductase by NKK-105, lipid peroxidation was decreased.

Jansson and Schenkman [18] demonstrated that lipid peroxidation cannot be detected in the liver microsomes of desaturase-induced rats. To account for the decrease of lipid peroxidation described in this paper, two possibilities can be considered. First, the activity of one pathway in the electron transport system is decreased by increasing the activity of another pathway, and second, the lipid composition of microsomes may be changed by the increase in desaturase activity. It is therefore possible that NKK-105 changes the lipid composition of liver microsomes. On the other hand, cytochrome P-450 content was not affected by the administration of NKK-105, while *N*-demethylase activities for aminopyrine, benzphetamine and ethylmorphine and *p*-nitroanisole *O*-demethylase activity were increased, and, conversely, aniline hydroxylase activity was decreased. In the reconstitution studies, benzphetamine *N*-demethylase [29] and *p*-nitroanisole *O*-demethylase [30, 31] activities are known to be increased by fortifying the cytochrome b_5. Therefore, it is likely that the increase of these demethylase activities *in vivo* described in this paper was due to the increase in cytochrome b_5 induced by NKK-105. On the other hand, it is possible that the composition of molecular species of cytochrome P-450 was changed by NKK-105.

In conclusion, the results presented here demonstrate that the induction pattern of the liver microsomal electron transport system shown by NKK-105 is characteristic and differs from those by the other inducers which have been reported in the past.

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